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EUROPEAN PATENT APPLICATION

⑲ Application number: 85105729.9

⑳ Date of filing: 10.05.85

⑤① Int. Cl.⁴: **A 61 K 37/36, C 12 N 11/04,**
A 61 D 7/00
// C12N15/00

③① Priority: 14.05.84 US 609924

④③ Date of publication of application: 21.11.85
Bulletin 85/47

⑥④ Designated Contracting States: **AT BE CH DE FR GB IT**
LI LU NL

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⑤④ Encapsulated mouse cells transformed with avian retrovirus-bovine growth hormone DNA, and a method of administering BGH in vivo.

⑤⑦ Recombinant DNA constructs having an avian retroviral long terminal repeat (LTR) ligated to the bovine growth hormone gene, were co-transformed into a mammalian cell (mouse) culture in order to obtain a stable cell culture secreting large amounts of bovine growth hormone. The transformed mouse cells were encapsulated in hollow fibers and implanted into animals, thereby producing circulating bovine growth hormone.

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TITLE OF THE INVENTION

ENCAPSULATED MOUSE CELLS TRANSFORMED WITH
AVIAN RETROVIRUS-BOVINE GROWTH HORMONE DNA, AND A
METHOD OF ADMINISTERING BGH IN VIVO

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BACKGROUND OF THE INVENTION

The present invention is directed to the use
of novel recombinant DNA molecules in which an avian
long terminal repeat (LTR) is ligated to the bovine
10 growth hormone (BGH) gene, and co-transformed into
stable mouse fibroblast cell lines containing the
recombinant genetic material integrated into the
mouse cell genome. These mouse cells can be
encapsulated into hollow fibers and implanted into
15 animals (e.g., subcutaneously, intradermally or
intraventricularly, etc.), thereby producing
circulating bovine growth hormone (BGH).

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SUMMARY OF THE INVENTION

Using recombinant DNA techniques, portions of the bovine growth hormone structural gene have been combined with eucaryotic regulatory regions of the Rous Sarcoma Viral genome to produce the materials herein designated as pBGH-3 and pBGH-4; these plasmids have been co-transformed into a mouse cell line; and the mouse cell line encapsulated into hollow fiber units and inserted into animals.

DETAILED DESCRIPTIONConstruction of Bovine Growth Hormone-Avian Retroviral DNA hybrids.

We have obtained bovine growth hormone genomic DNA in a pBR322 plasmid (pBGH-1). Using recombinant DNA technology, the bovine growth hormone promoter element was removed from the structural gene. This subcloned plasmid lacking a promoter was termed pBGH-2. We have ligated an avian retroviral promoter element, i.e., a viral Long Terminal Repeat (LTR), to the bovine growth hormone structural gene. Ligation was performed at 2 distinct restriction enzyme cleavage sites found within a region of each DNA which encodes 5'-untranslated portions of their m-RNAs. These 5'-untranslated portions of the m-RNAs ("leader" sequences) are nucleotides located between the m-RNA cap site and the translation initiation codon. Bovine growth hormone and avian retroviral 5'-untranslated nucleotide sequences are comprised of 59 base pairs and 379 base pairs, respectively.

These novel plasmid clones have been named pBGH-3 and pBGH-4.

Construction of Recombinant Plasmid, pBGH-3.

Plasmid DNAs (bovine growth hormone and Rous Sarcoma Virus) were transfected into E. coli RR1 cells. Clones were isolated and the plasmid DNAs
5 amplified. The resulting DNAs were characterized using diagnostic endodeoxyribonuclease recognition sites based on restriction enzyme maps available for the original clones. We have termed these plasmids pBGH-1 (plasmid bovine growth hormone-1) and pL397
10 (Rous Sarcoma Virus).

pBGH-1 (10 µg) was digested to completion with Bam HI and SalI. Two linear DNA fragments of µ5.0 kb and µ3.0 kb resulted. The fragments were separated by agarose gel electrophoresis (1%
15 Seaplaque agarose). The larger fragment containing the bovine growth hormone gene was eluted from the gel. To insure purity, this DNA again was subjected to agarose gel electrophoresis (1% Seaplaque) and elution. Approximately 500 ng was recovered.

20 Rous Sarcoma virus clone pL397 was enzymatically cleaved with BamHI and the five resulting linear DNA fragments separated by agarose gel electrophoresis (1% Seaplaque). A linear fragment of µ6.0 kb was eluted from the gel,
25 enzymatically digested with SalI, and subjected to a similar agarose gel electrophoresis procedure. Approximately 2.0 kb linear DNA fragment encoding the avian retroviral LTR (promoter) was purified. Approximately, 500 ng of DNA was recovered.

30 Ligation of the viral DNA sequences to the bovine growth hormone gene occurred at a BamHI site. Viral sequences include the 3' portion of the 'env' gene, the viral LTR, approximately 530 nucleotides of

the viral leader sequence and 5' portion of the viral 'gag' gene. Included in the bovine growth hormone segment of the recombinant plasmid are the 5 bovine growth hormone exons (shown in boxes) as well as μ 500
5 base pairs found at the 3' terminus of the gene.

100 ng of DNA containing equimolar quantities of the above isolated DNA fragments were ligated for 1 hour at 22°C. Following transfection into E. coli RR1 cells, 100 colonies were isolated.
10 Non-ligated control DNA preparations resulted in zero colonies.

Restriction enzymatic digestion analysis of this cloned DNA confirmed that the two segments of the above mentioned DNAs were joined, thus creating a
15 new biological molecule. This molecule has been termed pBGH-3.

Construction of Recombinant Plasmid, pBGH-4.

In order to alter pBGH-3 such that
20 non-essential regions of the Rous Sarcoma derived DNA were removed, the following procedure was followed.

A large 7.0 kb linear DNA fragment derived from pBGH-3 by BSTEII and BamHI cleavage was purified by agarose gel electrophoresis. Following excision
25 from the gel, the DNA was treated with E. coli DNA polymerase (Klenow large fragment) which resulted in the generation of molecules possessing flush termini. Blunt end ligation of these molecules and transfection into E. coli RR1 cells resulted in bacterial
30 clones containing plasmid DNA similar to that of pBGH-3. However approximately 430 base pairs between the viral BSTEII and BamHI sites have been deleted.

Restriction enzymatic digestion analysis of this cloned DNA confirmed that this non-essential region of the pBGH-3 had been removed. This plasmid has been designated pBGH-4.

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Sequencing of pBGH-4.

The DNA nucleotide sequencing of pBGH-4 was done in the following manner: pBGH-4- DNA (20 ng) was cleaved with EcoRI resulting in 3 linear DNA fragments of 4.3 kb, 2.15 kb, and 1.5 kb. The 2.15 kb DNA molecule was isolated by agarose gel electrophoresis (1% Seaplaque agarose). Following elution of the DNA from the agarose gel, the 5' protruding termini were labeled by addition of α -5' (32 P) dATP using E. coli DNA polymerase I (Klenow fragment). The 32 P labeled DNA molecule was cleaved subsequently with SmaI yielding 2 DNA molecules of 2.0 kb and 0.5 kb in size. The DNA molecule containing 2.0 kb was purified by agarose gel electrophoresis (1% Seaplaque agarose) and sequenced according to the procedure of Maxam and Gilbert, Proc. Natl. Acad. Sci. USA, 74: 560-564 (1977). The junction between the RSV-LTR and the bovine growth hormone gene was confirmed to be the predicted sequence:

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| | LTR | | BGH |
|----|-----------------------|---------------------|-----|
| 5' | C A T T T G G T G A C | G A T C C C A G G A | 3' |
| 3' | G T A A A C C A C T G | C T A G G G T C C T | 5' |

Transient Assay for Screening Plasmid Constructs.

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We have also developed a rapid and sensitive transient assay system for the detection of bovine

growth hormone by cultured rat GH3 cells transfected with plasmid DNA encoding bovine growth hormone, to quickly screen a variety of plasmid constructs for their ability to direct the synthesis of BGH. We
5 have shown and confirmed that pBGH-4 directs the synthesis and secretion of bovine growth hormone by these cells.

The optimum conditions for the transient expression of bovine growth hormone by rat GH3 cells
10 have been determined.

It has been reported in the literature that DNA transfection of cultured cells mediated by DEAE-Dextran is influenced by at least two parameters. They are (1) the concentration of DNA used in the
15 experiment and (2) the length of time cells are exposed to the DEAE-Dextran-DNA complex. We have optimized our transient assay system in rat GH3 relative to these two parameters.

Briefly about 5.0×10^5 rat GH3 cells were
20 plated onto 35 mm tissue culture plates. Following overnight incubation, cells were rinsed with 2.0 ml culture fluid minus serum. 1.0 ml of culture fluid minus serum containing a variety of concentrations of pBGH-4 DNA along with DEAE-Dextran (200 $\mu\text{g}/\text{ml}$) was
25 added to the cells. Following incubation at 37°C for a variety of time intervals, the DEAE-Dextran-DNA solution was removed and the cells rinsed two times with complete medium. Cells were incubated for 5 days with changes in cultured fluid at daily
30 intervals. Bovine growth hormone was assayed in the culture fluid using a sensitive radioimmunoassay.

Using this assay, pBGH-4 directed detectable levels of bovine growth hormone, detectable at 48

hours and 72 hours post transfection. Increasing amount of DNA in the DEAE-DNA complex used per transfection resulted in a respective elevation in BGH secretion. At 72 hours, cells exposed to 500 ng of DNA produced approximately 10 fold more (283.8 ng/ml) BGH relative to these exposed to 250 ng DNA, (30 ng/ml).

In a similar manner the amount of time to which the cells were exposed to the DEAE-Dextran-DNA mixture was altered. In this experiment, 250 ng of DNA was used in each transfection. Bovine growth hormone expression varied inversely with the duration of transfection time.

From these and other related experiments, and although results do vary from one set of experiments to another, it is concluded that pBGH-4 DNA directs the synthesis of detectable levels of bovine growth hormone by cultured rat GH3 cells following DEAE-Dextran mediated transfection.

The optimum amount of DNA used in these transfection experiments ranged from 250 ng to 500 ng per 5×10^5 cells.

Time intervals of DEAE-Dextran-DNA exposure to GH3 cells between 30 minutes and 45 minutes are optimal for subsequent expression of bovine growth hormone.

Production of Mouse Cell Lines Secreting Bovine Growth Hormone.

In order to produce a large amount of purified BGH protein, we generated cultured mouse fibroblasts secreting the BGH. This was performed by

co-transforming mouse TK(-) L cells with Herpes Viral TK DNA and plasmid vectors capable of directing BGH expression as determined by our transient assay.

After selecting for a TK(+) phenotype, plasmids
5 encoding BGH will be present and active in directing the expression of Bovine Growth Hormone.

To 5×10^5 mouse L cells (LTK⁻, APRT⁻)
was added a complex of calcium phosphate precipitated
DNA. Included in the complex was 10 µg of L cell
10 (LTK⁻, APRT⁻) DNA, 10 µg of pBGH-4 DNA, and 100
ng of pTK5 DNA. Ten TK positive colonies were
selected and subcultured. The amount of bovine
growth hormone secreted into the culture fluid was
determined. Culture L-BGH-4-3 is secreting about 3.0
15 µg BGH per 5×10^6 cells, per 24 hours.

In order to generate a mouse cell line which
secrete a larger quantity of BGH, an alternative
approach was performed. The protocol involves
cotransformation of mouse-L-cells (TK⁻, APRT⁻) by a
20 plasmid DNA which encodes the APRT gene and a
truncated TK gene, PdLAT-3, (Robert & Axel, 1982)
along with plasmid pBGH-4 DNA. Results from these
types of cotransformation experiments have revealed
that plasmid DNA involved in the cotransformation are
25 amplified within the mouse cell following trans-
fection. Amplification of the plasmid DNA results in
a corresponding amplification of gene product ().

Briefly, 5×10^5 cells are cotransformed,
using the CaPO₄ method above, with PdLAT3 (20 ng)
30 and BGH-4 (2 µg) DNA. The cells are first selected
in DME plus 10% CS plus 4 µg/ml azaserine, plus 15
µg/ml adenine for selection of the APRT⁺ phenotype.

These APRT⁺ cells are then selected in DME plus 10% CS plus 15 µg/ml hypoxanthine plus 1 µg/ml aminopterin plus 5.15 µg/ml thymidine for selection of the TK⁺ phenotype. The APRT⁺, TK⁺ cells are subsequently
5 screened for their ability to secrete BGH. Twenty stable positive colonies were generated and sub-cultured. The amount of BGH secreted into the culture fluid was determined. Culture L-BGH-4-13 is secreting 75 µg BGH per 5 X 10⁶ cells per 24 hours.

10 BGH produced by these cells can be purified as known in the art and used as designed, e.g., as a growth stimulant in animals, see EPO 0085036, Monsanto, and EPO 0068646, Upjohn.

Two mouse L-cell lines stably transformed
15 with pBGH-4 DNA and expressing large amounts of BGH as well as plasmids pBGH-3 and pBGH-4 have been deposited at the American Type Culture Collection, Rockville. The deposit numbers are ATCC CRL 8536 and ATCC CRL-8537, for the mouse cell cultures
20 BGH-4-13 and BGH-4-3, respectively, deposited April 6, 1984; and ATCC 39674 and ATCC 39675, for pBGH-3 and pBGH-4, respectively, deposited May 1, 1984.

The highly important use of the mouse cell
25 lines L-BGH-4-3 and L-BGH-4-13 secreting bovine growth hormone is in a hollow fiber delivery system, which can be implanted into many commercially important animal species, such as chickens, turkeys, cattle, swine, and sheep.

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In vitro Expression of Bovine Growth Hormone by Mouse Cells Encapsulated in a Hollow Fiber Unit.

- The mouse cell line L-BGH-4-3 described above was loaded into a hollow fiber (polyvinyl-chloride-acrylic copolymer, XM-50 manufactured and sold by Amicon). This fiber has a 1100 μ m internal diameter and is approximately 10 mm long. The Scanning Electron Micrograph (SEM) of the fiber, see Hymer et al., Neuroendocrinology, 32: 339-349 (1981), "Pituitary Hollow Fiber Units in vivo and in vitro," shows the spongy layer of polymer with increasing openings to one fiber periphery. Each fiber unit was loaded with 7-8 μ l of a mouse cell suspension (L-pBGH-4-3) containing about 250,000 cells/ μ l.
- Following encapsulation, the ends of the hollow fiber were sealed and incubated in 35 mm culture dishes with DMED medium. Media were collected at various time intervals and analyzed for BGH by a BGH RIA. BGH was detected in the culture fluid 48 hours after the capsules were plated in culture dishes. The cells within the fiber were secreting BGH 75 days post encapsulation. The amounts of BGH measured by RIA are in direct correlation with the number of capsules incubated.
- This is a novel in vitro system in which eucaryotic cells producing BGH can be analyzed by using these hollow fibers.
- The fiber-mouse cell line L-BGH-4-3 system could be used to produce and purify BGH simply by allowing the cells to secrete GH through the fiber filter unit into an appropriate medium.

The fibers could also usefully be implanted into animals. BGH has been shown to stimulate milk production in dairy cattle, see Machlin, J. Dairy Sci., Vol 56, No. 5, p. 575; C. J. Peel, et al., J. Nutr. (1981), 111 (9) 1662-1671; see also EPO application 85036, published August 3, 1983.

When these fibers are implanted into poultry such as chickens, and small mammals, such as rats, BGH is detected in the circulatory system.

The fibers can also be implanted, either subcutaneously, intradermally or intraventricularly, etc., into large animals, especially those valuable as milk producers, such as dairy cows. An effective amount of BGH to stimulate milk production is 0.005 to 200,000 µg per animal per day, and this amount can be produced by selected fiber units containing the BGH-producing mouse cell line. See also EPO 0085036, herein incorporated by reference, for amounts and regime of administration.

In addition to the specific example in this application, using one encapsulated mouse cell line cotransformed with bovine growth hormone gene, to produce BGH when implanted, it will be apparent to those skilled in the art that any eucaryotic cells transfected with biologically active DNA and secreting corresponding proteins can be put into the polyvinylchloride-acrylic copolymer fibers described. Specifically, any of the BGH expression systems claimed in GB 2073245 (Yeda), EPO 0085 036 (Monsanto), EPO 0067 026 (Michigan State), or EPO 0068 646 (Upjohn) can be inserted into the mouse cell

line herein described, or any other suitable cell line, encapsulated in the fiber; and implanted in any animal.

Also, other cell lines producing other
5 hormones or proteins can be encapsulated in these fibers and used as herein described. Even more generally, any eucaryotic cell can be transfected with biologically active DNA, the particular cell selected and cloned for production, expression and
10 secretion of the corresponding protein product; and these cells encapsulated as herein described. Examples of other proteins expressed by cell lines to which the methodology of this invention is applicable include human serum albumin, human interferons, human
15 antibodies, human insulin, blood clotting factors, human growth factors, brain peptides, enzymes, hormones, prolactin, viral antigens and plant proteins.

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WHAT IS CLAIMED IS:

1. The method for increasing milk
production in cows by in vivo implantation in said
5 cows of an effective amount of the encapsulated cell
line L-BGH-4-3, ATCC CRL 8537 or L-BGH-4-13, ATCC
CRL-8536.
2. The method for increasing animal growth
10 by implantation in vivo in said animals an effective
amount of the encapsulated cell line L-BGH-4-3, ATCC
CRL 8537 or L-BGH-4-13, ATCC CRL-8536.
3. The methods of Claims 1 or 2 in which
15 the encapsulated cell line is implanted
subcutaneously.
4. A hollow fiber, about 10 mm long
polyvinyl chloride-acrylic copolymer, having about a
20 1100 μ m internal diameter, containing a recombinant
mouse cell suspension capable of producing bovine
growth hormone in an amount sufficient to stimulate
animal growth or milk production when implanted in an
animal.
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5. A hollow fiber, about 10 mm long,
polyvinylchloride-acrylic copolymer, having about a
1100 μ m internal diameter, containing a cell
suspension which has been previously transfected with
30 biologically active DNA, said cells having been
selected for production and secretion of the

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corresponding protein product, the cell suspension
thusly being capable of producing the protein in a
biologically active amount when implanted in an
animal.

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